ANTISENSE MODULATION OF EXPRESSION OF TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTORS (TRAFs)

BACKGROUND

This application is is a continuation of U.S. Patent Application No. 09/167,109 filed October 6, 1998.

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of tumor necrosis factor receptor-associated factors (TRAFs). In particular, this invention relates to antisense compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding human TRAFs. Such oligonucleotides have been shown to modulate the expression of TRAFs.

BACKGROUND OF THE INVENTION

Tumor necrosis factor (TNF) receptor superfamily members regulate cellular proliferation, differentiation and apoptosis in inflammatory and immune responses. This receptor superfamily comprises a group of related cell-surface receptors including, but not limited to, types 1 and 2 TNF receptors (TNFR1 and TNFR2), Fas, CD27, 4-1BB, CD40 and CD30. Signaling through TNF receptor superfamily members is initiated by oligomerization of the receptors with trimeric ligands, bringing intracellular domains in close proximity (Pullen et al., *Biochemistry* **1998**, 37, 11836-11845). Two families of adaptor proteins that associate with TNF receptor superfamily members have been

identified: the TNF receptor-associated factor (TRAF) family, and the death domain-containing protein family.

Members of the TRAF family of proteins share an amino-terminal RING finger motif and a homologous carboxy-terminal region, referred to in the art as the TRAF domain (Yuan, J., *Curr. Opin. Cell Biol.* **1997**, 9, 247-251. This conserved carboxy-terminal region binds to receptor cytoplasmic domains and mediates interactions with the signaling proteins NF-κB inducing kinase (NIK) and I-TRAFT/TANK (Cheng et al., *Science* **1995**, 267, 1494-1498; Cheng, G. and Baltimore, D., *Genes Dev.* **1996**, 10, 963-973; Rothe et al., *Proc. Natl Acad. Sci. USA* **1996**, 93, 8241-8246; Malinin et al., *Nature* **1997**, 385, 540-544). A predicted coiled-coil region mediating TRAF homo- and hetero-oligomerization is in a less conserved region N-terminal to the TRAF domain (Cao et al., *Nature* **1996**, 383, 443-446; Cheng et al., *Science* **1995**, 267, 1494-1498; Rothe et al., *Cell* **1994**, 78, 681-692; Sato et al., *FEBS Lett* **1995**, 358, 113-118; and Takeuchi et al., *J. Biol. Chem* **1996**, 271, 19935-19942).

The mammalian TRAF family currently includes six members, TRAF-1, TRAF-2, TRAF-3, TRAF-4, TRAF-5 and TRAF-6. These proteins have generally been found within the cytosols of cells, either in association with cytosolic vesicles or at the plasma membrane after addition of selected TNF family cytokines to the cells. Members of the TRAF family mediate signals for various different receptors. Subsets of TRAF family members have been shown to interact with the TNF receptor family members (TNFR2, CD40, CD30, LTβR, ATAR, OX40 and 4-1BB).

For example, TRAF-1 and TRAF-2 were identified by their ability to interact with the cytoplasmic domains of TNFR2 (Rothe et al., *Cell* **1994**, 78, 681-691). TNFR2 has been associated with TNF's ability to stimulate cell

proliferation and activation of NFκB (Tartaglia et al., *Proc. Natl Acad. Sci. USA* **1991**, 88, 9292-9296). TRAF-1 is believed to be involved in the regulation of apoptosis (Speiser et al., *J. Exp. Med.* **1997**, 185, 1777-1783). Depletion of TRAF-2 and its co-associated proteins has also been shown to increase the sensitivity of the cell to undergo apoptosis during activation of death inducing receptors such as TNFR1 (Duckett, C.S. and Thompson, C.B., *Genes & Development* **1997**, 11, 2810-2821; Yeh et al., *Immunity* **1997**, 7, 715-725). Accordingly, the rate of receptor-mediated TRAF-2 consumption and TRAF-2 translation has been suggested to play a dynamic role in the regulation of cell survival (Duckett, C.S. and Thompson, C.B., *Genes & Development* **1997**, 11, 2810-2821). Targeted disruption of the TRAF-2 gene in mice has also been shown to generate severe defects in c-Jun N-terminal kinase (JNK) activation through TNFR1 (Yeh et al., *Immunity* **1997**, 7, 715-725).

TRAF-2 (Rothe et al., *Science* **1995**, 269, 1424-1427), TRAF-3 (Cheng et al., *Science* **1995**, 267, 1494-1498), TRAF-5 (Ishida et al., *Proc. Natl Acad. Sci USA* **1996**, 93, 9437-9442) and TRAF-6 (Pullen et al., *Biochemistry* **1998**, 37, 11836-11845) have also been shown to interact with the B lymphocyte receptor CD40. CD40 is a TNF receptor superfamily member that provides activation signals in antigen presenting cells such as B cells, macrophages and dendritic cells. Activation of CD40 leads to B-cell survival, growth and differentiation. In 293T cells, expression of TRAF-3 suppressed constitutive activity of NFκB, whereas expression of TRAF-5 induced NFκB activity. Targeted disruption of the TRAF-3 gene in mice causes impaired immune responses to T-dependent antigens and results in early postnatal lethality (Xu et al., *Immunity* **1996**, 5, 407-415). TRAF-2, TRAF-5 or TRAF-6 overexpression in mammalian cells also induces JNK activation.

TRAF-4 is expressed in breast cancers. In *in vitro* binding assays, TRAF-4 has been shown to interact with the cytosolic domain of the lymphotoxin-β receptor (LTβR) and weakly with the p75 nerve growth factor receptor but not with TNFR1, TNFR2, Fas or CD40 (Karjewska et al., *Am. J. of Pathol.* **1998**, 152, 6, 1549-1561).

TRAF-6 has also been reported to mediate the signal transduction pathway induced by IL-1 to activate NFκB by recruiting IL-1 receptor associated kinase (IRAK), a serine/threonine kinase (Cao et al., *Nature* **1996** 93:9437-9442). Thus, the role of TRAFs extends beyond being signal transducers for the TNF-receptor superfamily.

The TRAF-5 protein and DNA encoding TRAF-5 are disclosed in WO97/38099. Also disclosed in WO97/38099 is an antisense oligonucleotide against the DNA, an anti-TRAF-5 antibody, a vector containing the DNA, transformants containing this vector and methods of producing TRAF-5 with this vector. In addition, this PCT application discloses methods of screening substances binding to TRAF-5 and substances regulating the activity and expression of this protein.

A TRAF family molecule, a polynucleotide coding for this molecule, an antibody against the molecule and an antisense polynucleotide of the molecule are also disclosed in WO97/31110. Disclosed in this PCT application are the base sequence of the gene and the amino acid of this "unknown" TRAF family molecule, which in addition to the antibody, are suggested to provide means for elucidating the functions of the proteins and the signal transducer system of a TNF-R family in which this molecule participates, to provide probes for research and diagnosis, and to indicate applications for therapeutic agents.

Currently, however, there are no known therapeutic agents which effectively inhibit the synthesis of one or more selected TRAF family members. Consequently, there is a

long-felt need for agents capable of effectively inhibiting TRAF expression. Antisense oligonucleotides against one or more TRAFs may therefore prove to be uniquely useful in a number of therapeutic, diagnostic and research applications.

SUMMARY OF THE INVENTION

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding a selected tumor necrosis factor receptor-associated factor (TRAF), and which modulate the expression of the selected TRAF. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating the expression of TRAFs in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of a selected TRAF by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding selected tumor necrosis factor receptor-associated factors (TRAFs), ultimately modulating the amount of the selected TRAF produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding the selected TRAF. By "selected TRAF" it is meant any member of the TRAF family of

proteins, most preferably TRAF-1, TRAF-2, TRAF-3, TRAF-4, TRAF-5 or TRAF-6. As used herein, the terms "target nucleic acid" and "nucleic acid encoding TRAF" encompass DNA encoding a TRAF family member, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of the selected TRAF. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multi-step process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is

one or more nucleic acid molecules encoding one or more selected TRAFs. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding a TRAF, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, *i.e.*, 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA,

respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns", which are excised from a transcript before it is translated. The remaining (and therefore

translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, *i.e.*, intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, *i.e.*, hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing

such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimens in cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (*i.e.*, from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2′, 3′ or 5′ hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to

form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3′ to 5′ phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3′-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3′-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3′-5′ linkages, 2′-5′ linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3′-5′ to 5′-3′ or 2′-5′ to 5′-2′. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897;

5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has

been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. Patent 5,489,677, and the amide backbones of the above referenced U.S. Patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides

comprise one of the following at the 2′ position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2′-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in United States patent application Serial Number 09/016,520, filed on January 30, 1998, which is commonly owned with the instant application and the contents of which are herein incorporated by reference.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2′-5′ linked oligonucleotides and the 5' position of 5' terminal nucleotides. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053;

5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is incorporated herein by reference.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-Me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Patent 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, *J.I., ed.* John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Crooke, S.T. and Lebleu, B. eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 289-302. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines,

including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, U.S.: 3,687,808; 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,681,941; and 5,750,692, each of which is herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA,* **1989**, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, 4, 1053-1060), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, 20, 533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, 259, 327-330; Svinarchuk et al., *Biochimie*, **1993**, 75, 49-54), a phospholipid, *e.g.*, di-

hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, 277, 923-937.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the

context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355;

5,652,356; and 5,700,922, each of which is herein incorporated by reference.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon

administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 or in WO 94/26764.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, 66, 1-19).

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, *etc.;* (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid,

p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of one or more members of the TRAF family is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, *e.g.*, to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding one or more selected TRAFs, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding one or more TRAFs can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of TRAF in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or

systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, *e.g.*, by inhalation or insufflation of powders or aerosols, including by nebulizer; (intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

For example, pharmaceutical compositions and/or formulations comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33). One or more penetration enhancers from one or more of these broad categories may be included.

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8, 2, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1, 1-33; El-Hariri et al., *J. Pharm. Pharmacol.*, **1992**, 44, 651-654). Examples of some presently preferred fatty acids are sodium caprate and sodium laurate, used singly or in combination at concentrations of 0.5 to 5%.

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, **1996**, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally

occurring components of bile as well as any of their synthetic derivatives.

Regardless of the method by which the antisense compounds of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the compounds and/or to target the compounds to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., *Current Op. Biotech.*, **1995**, 6, 698-708).

Liposome preparation is described in pending United States patent application 08/961,469, filed on October 31, 1997, which is commonly owned with the instant application and which is herein incorporated by reference.

Certain embodiments of the invention provide for liposomes and other compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites are purchased from commercial sources (*e.g.*, Chemgenes, Needham, MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides is utilized, except the wait step after pulse delivery of tetrazole and base is increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods (Sanghvi, et. al., *Nucleic Acids Research*, **1993**, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham, MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides are synthesized as described previously by Kawasaki, et. al., *J. Med. Chem.*, **1993**, 36, 831-841 and U.S. Patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine are synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine is selectively protected in moderate yield as the 3',5'-

ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups is accomplished using standard methodologies and standard methods are used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2′-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine is accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group is followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation is followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies are used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2′-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine is accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil is treated with 70% hydrogen fluoride-pyridine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2′-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine is synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2′-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites were prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum dried in a vacuum oven (60°C at 1 mm Hg for 24 hours) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel

and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/Acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 hours using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was

stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) is added with stirring. The mixture was stirred for 3 hours (tlc showing the reaction to be approximately 95% complete). The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was then dissolved in $CHCl_3$ (700 mL) and

extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showing the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-(Aminooxyethyl) nucleoside amidites and 2'-(dimethylaminooxyethyl) nucleoside amidites

Aminooxyethyl and dimethylaminooxyethyl amidites are prepared as per the methods described in United States patent applications serial number 10/037,143, filed February 14, 1998, and serial number 09/016,520, filed January 30, 1998, each of which is herein incorporated by reference.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) were synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 seconds and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hours), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait

step after the delivery of tetrazole and base to 600 seconds repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 Ammonia/Ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hours at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hours at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxy-ethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2′-O-methyl chimeric oligonucleotide, with the substitution of 2′-O-(methoxyethyl) amidites for the 2′-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides were prepared as per the above procedure for the 2′-O-methyl chimeric oligonucleotide with the substitution of 2′-O-(methoxyethyl) amidites for the

2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides were purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides are purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, 266, 18162-18171. Results obtained with HPLC-purified material are similar to those obtained with non-HPLC purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences

simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (*e.g.* PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides were synthesized as per known literature or patented methods. They were utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples were then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (*e.g.*, Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR, RNAse protection assay (RPA) or Northern blot analysis. The following four cell types are provided for illustrative purposes, but other cell types can be routinely used.

T-24 cells:

The transitional cell bladder carcinoma cell line T-24 is obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells are routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells are routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells are seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 is obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells are routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies,

Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells are routinely passaged by trypsinization and dilution when they reach 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) are obtained from the Clonetics Corporation (Walkersville MD). NHDFs are routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville, MD) supplemented as recommended by the supplier. Cells are maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) are obtained from the Clonetics Corporation (Walkersville, MD). HEKs are routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville, MD) formulated as recommended by the supplier. Cells are routinely maintained for up to 10 passages as recommended by the supplier.

Treatment with antisense compounds:

When cells reach 80% confluency, they are treated with oligonucleotide. For cells grown in 96-well plates, wells are washed once with 200 µL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after oligonucleotide treatment.

Example 10

Analysis of oligonucleotide inhibition of TRAF expression

Antisense modulation of TRAF expression can be assayed in a variety of ways known in the art. For example, TRAF mRNA levels can be quantitated by, *e.g.*, Northern blot analysis, RNAse protection assay (RPA), competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 1, John Wiley & Sons, Inc., **1993**, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 1, John Wiley & Sons, Inc., **1996**, pp. 4.2.1-4.2.9. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

TRAF protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to TRAF can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1997**, pp. 11.12.1-11.12.9. Preparation of monoclonal antibodies is taught in, for example, Ausubel, et al., *Current Protocols*

in Molecular Biology, Volume 2, John Wiley & Sons, Inc., **1997**, pp. 11.4.1-11.11.5.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1998**, pp. 11.4.1-11.11.5. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1997**, pp. 10.8.1-10.8.21. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1991**, pp. 11.2.1-11.2.22.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA is isolated according to Miura et al., *Clin. Chem.*, **1996**, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 1, John Wiley & Sons, Inc., **1993**, pp. 4.5.1-4.5.3. Briefly, for cells grown on 96-well plates, growth medium is removed from the cells and each well is washed with 200 µL cold PBS. 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) is added to each well, the plate is gently agitated and then incubated at room temperature for 5 minutes. 55 µL of lysate is transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine, CA). Plates are incubated for 60 minutes at room temperature, washed 3 times with 200 µL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate is blotted on paper towels to remove excess wash buffer and

then air-dried for 5 minutes. 60 µL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C is added to each well, the plate is incubated on a 90°C hot plate for 5 minutes, and the eluate is then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia, CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 100 µL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 µL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute,

and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

Example 13

Real-time Quantitative PCR Analysis of TRAF mRNA Levels

Quantitation of TRAF mRNA levels is determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (*e.g.*, JOE or FAM, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (*e.g.*, TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved

from their respective probes, and the fluorescence intensity is monitored at regular (six-second) intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents are obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions are carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM $MgCl_2$, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL poly(A) mRNA solution. The RT reaction is carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol are carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension). TRAF probes and primers are designed to hybridize to the human TRAF sequence, using published sequence information. For example, GenBank Accession No. U19261, Locus name "HSU 19261" SEQ ID NO: 1; GenBank Accession No. U12597, Locus name "HSU12597" SEQ ID NO. 2; GenBank Accession No U21092, Locus name "HSU21092 SEQ ID NO: 3; GenBank Accession No. X80200, Locus name "HSMLN62" SEQ ID NO. 4; GenBank Accession No. AB000509, Locus name "AB000509 SEQ ID NO. 5; GenBank Accession No. U78798, Locus name "HSU78798" SEQ ID NO. 6.

Example 14

Antisense inhibition of TRAF-1 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human TRAF-1 RNA, using published sequences (GenBank accession number U19261, incorporated herein as SEQ ID NO: 1). The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. U19261), to which the oligonucleotide binds. All compounds in Table 1 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout.

TABLE 1

Nucleotide Sequences of Human TRAF-1 Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
26698	TTTAAGTTGCTCCAGGGC	7	0028-0045	5'-UTR
26699	GCCGGGCGAGGACTGCTG	8	0093-0110	coding
26700	GCAGACGGTGGGAGGGCA	9	0139-0156	coding
26701	CTGGGCTCCTTTGGGTCC	10	0159-0176	coding
26702	CACAGCAGAGAGCCCTGG	11	0173-0190	coding
26703	ATTCCTCGGGTTCTCAGA	12	0202-0219	coding
26704	CCATTCCTCGGGTTCTCA	13	0204-0221	coding
26705	CCTCGCCATTCCTCGGGT	14	0209-0226	coding
26706	GATCCTCGCCATTCCTCG	15	0212-0229	coding
26707	AGACGGCTTCCTGGGCTT	16	0270-0287	coding
26708	TTGAAGGAGCAGCCGACA	17	0351-0368	coding
26709	GGCCTTCCACTGTTTCAT	18	0442-0459	coding

26710	CCACTTCCACGGCTGCCT	19	0527-0544	coding
26711	CGCCTGGTGACATTGGTG	20	0894-0911	coding
26712	CGCATCATACTCCCCTCT	21	1063-1080	coding
26713	AGGCGTCAATGGCGTGCT	22	1142-1159	coding
26714	GGAAGGCGTCAATGGCGT	23	1145-1162	coding
26715	GGAAGAAGAGTGGGCATC	24	1223-1240	coding
26716	CGTAGGCGTGCTTGGGTG	25	1259-1276	coding
26717	GCCCCGCCCACCCTAAGT	26	1321-1338	stop
26718	GGAGCCCCGCCCACCCTA	27	1324-1341	stop
26719	CTCAGGAGCCCCGCCCAC	28	1328-1345	3'-UTR
26720	AAGGGCAGGGCATCACAG	29	1380-1397	3'-UTR
26721	TTTGTGCCCTGAGGTCTT	30	1405-1422	3'-UTR
26722	CACCCATCTTTGTGCCCT	31	1413-1430	3'-UTR
26723	GGCCTCCCAGTGTCGCAT	32	1570-1587	3'-UTR
26724	CCCGGTCCTGTTTCTGAC	33	1756-1773	3'-UTR
26725	GCACCCCATCCCTTCCAC	34	1773-1790	3'-UTR
26726	TGGAGCCGTCTGGGTTTG	35	1837-1854	3'-UTR
26727	GTCTTCAAATCCAACCCC	36	1871-1888	3'-UTR
26728	TTCTGGGCTGGAAGGAAA	37	1896-1913	3'-UTR
26729	ACTTTCTGGGCTGGAAGG	38	1899-1916	3'-UTR
26730	AGAGACTTTCTGGGCTGG	39	1903-1920	3'-UTR
26731	TTTCCAGAACCCCTGTAG	40	1955-1972	3'-UTR
26732	ATGTTTCCAGAACCCCTG	41	1958-1975	3'-UTR
26733	GGGCTGGGTGTGCTCCTG	42	2090-2107	3'-UTR
26734	TTTATGCCCCTCTTCTTC	43	2204-2221	3'-UTR
26735	GGAAAGTTTATGCCCCTC	44	2210-2227	3'-UTR
26736	TACGGGATTCTGGAAAGC	45	2257-2274	3'-UTR
26737	AGGTGTTACGGGATTCTG	46	2263-2280	3'-UTR

[1] All cytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

[2] Coordinates from GenBank Accession No. U19261, locus name "HSU19261" SEQ ID NO.1.

Example 15:

Antisense inhibition of TRAF-1 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human TRAF-1 were synthesized. The oligonucleotide sequences are shown in Table 2. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. U19261), to which the oligonucleotide binds.

All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

TABLE 2

Nucleotide Sequences of Human TRAF-1 Gapmer Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
26738	**TTTA**AGTTGCTCCA**GGGC**	7	0028-0045	5'-UTR
26739	**GCCG**GGCGAGGACT**GCTG**	8	0093-0110	coding
26740	**GCAG**ACGGTGGGAG**GGCA**	9	0139-0156	coding
26741	**CTGG**GCTCCTTTGG**GTCC**	10	0159-0176	coding

26742	**CACA**GCAGAGAGCC**CTGG**	11	0173-0190	coding
26743	**ATTC**CTCGGGTTCT**CAGA**	12	0202-0219	coding
26744	**CCAT**TCCTCGGGTT**CTCA**	13	0204-0221	coding
26745	**CCTC**GCCATTCCTC**GGGT**	14	0209-0226	coding
26746	**GATC**CTCGCCATTC**CTCG**	15	0212-0229	coding
26747	**AGAC**GGCTTCCTGG**GCTT**	16	0270-0287	coding
26748	**TTGA**AGGAGCAGCC**GACA**	17	0351-0368	coding
26749	**GGCC**TTCCACTGTT**TCAT**	18	0442-0459	coding
26750	**CCAC**TTCCACGGCT**GCCT**	19	0527-0544	coding
26751	**CGCC**TGGTGACATT**GGTG**	20	0894-0911	coding
26752	**CGCA**TCATACTCCC**CTCT**	21	1063-1080	coding
26753	**AGGC**GTCAATGGCG**TGCT**	22	1142-1159	coding
26754	**GGAA**GGCGTCAATG**GCGT**	23	1145-1162	coding
26755	**GGAA**GAAGAGTGGG**CATC**	24	1223-1240	coding
26756	**CGTA**GGCGTGCTTG**GGTG**	25	1259-1276	coding
26757	**GCCC**CGCCCACCCT**AAGT**	26	1321-1338	stop
26758	**GGAG**CCCCGCCCAC**CCTA**	27	1324-1341	stop
26759	**CTCA**GGAGCCCCGC**CCAC**	28	1328-1345	3'-UTR
26760	**AAGG**GCAGGGCATC**ACAG**	29	1380-1397	3'-UTR
26761	**TTTG**TGCCCTGAGG**TCTT**	30	1405-1422	3'-UTR
26762	**CACC**CATCTTTGTG**CCCT**	31	1413-1430	3'-UTR
26763	**GGCC**TCCCAGTGTC**GCAT**	32	1570-1587	3'-UTR
26764	**CCCG**GTCCTGTTTC**TGAC**	33	1756-1773	3'-UTR
26765	**GCAC**CCCATCCCTT**CCAC**	34	1773-1790	3'-UTR
26766	**TGGA**GCCGTCTGGG**TTTG**	35	1837-1854	3'-UTR
26767	**GTCT**TCAAATCCAA**CCCC**	36	1871-1888	3'-UTR
26768	**TTCT**GGGCTGGAAG**GAAA**	37	1896-1913	3'-UTR
26769	**ACTT**TCTGGGCTGG**AAGG**	38	1899-1916	3'-UTR
26770	**AGAG**ACTTTCTGGG**CTGG**	39	1903-1920	3'-UTR

26771	**TTTC**CAGAACCCCT**GTAG**	40	1955-1972	3'-UTR
26772	**ATGT**TTCCAGAACC**CCTG**	41	1958-1975	3'-UTR
26773	**GGGC**TGGGTGTGCT**CCTG**	42	2090-2107	3'-UTR
26774	**TTTA**TGCCCCTCTT**CTTC**	43	2204-2221	3'-UTR
26775	**GGAA**AGTTTATGCC**CCTC**	44	2210-2227	3'-UTR
26776	**TACG**GGATTCTGGA**AAGC**	45	2257-2274	3'-UTR
26777	**AGGT**GTTACGGGAT**TCTG**	46	2263-2280	3'-UTR

[1] Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines and 2'-deoxycytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

[2] Coordinates from GenBank Accession No. 19261, locus name "HSU19261" SEQ ID NO. 1.

Example 16:

Antisense inhibition of TRAF-2 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a series of oligonucleotides targeted to human TRAF-2 were synthesized. The oligonucleotide sequences are shown in Table 3. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. U12597), to which the oligonucleotide binds.

All compounds in Table 3 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of eight 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by six-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) in the central "deoxy gap" and phosphodiester (P=O) in the wings.

Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

TABLE 3:

Nucleotide Sequences of TRAF-2 Gapmer Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
16827	GoToCoGoCoAsGsCsGsCsGsCsCsGsGoAoAoToToC	47	0001-0020	5'-UTR
16828	CoCoAoAoCoGsGsTsCsGsCsAsGsCsGoCoGoCoCoG	48	0007-0026	5'-UTR
16829	CoAoGoCoCoAsTsGsAsGsAsGsCsTsGoToGoAoCoC	49	0042-0061	AUG
16830	AoCoGoCoToAsGsCsTsGsCsAsGsCsCoAoToGoAoG	50	0052-0071	AUG
16831	GoCoCoAoCoAsCsTsGsCsGsCsCsTsGoGoAoAoGoG	51	0185-0204	coding
16832	CoCoGoGoCoAsGsGsCsTsCsTsCsCsAoCoCoToCoC	52	0348-0367	coding
16833	GoCoAoGoCoGsGsCsCsTsTsCsGsTsGoGoCoAoGoC	53	0422-0441	coding
16834	CoCoToCoGoTsGsGsTsGsCsGsCsCsToToCoAoCoG	54	0576-0595	coding
16835	CoToCoGoAoCsAsCsTsTsGsCsCsAsCoAoAoGoToC	55	0675-0694	coding
16836	CoAoCoToGoCsAsCsCsTsCsGsTsGsCoToCoCoToG	56	0751-0770	coding
16837	CoCoToCoToGsCsAsGsGsAsGsCsTsCoToGoAoCoC	57	0848-0867	coding
16838	CoAoGoCoCoGsGsTsGsCsTsGsCsCsGoGoCoToGoC	58	0962-0981	coding
16839	CoCoGoGoToGsCsCsGsTsCsGsCsCsGoToToCoAoG	59	1240-1259	coding
16840	AoCoGoToCoGsGsGsCsCsTsGsAsAsGoGoCoGoToC	60	1387-1406	coding

16841	**C**o**T**o**G**o**T**o**C**o**A**sGsGsTsCsCsAsCsAs**A**o**T**o**G**o**G**o**C**o**C**	61	1533-1552	coding
16842	**G**o**C**o**C**o**G**o**G**o**C**sTsGsTsGsCsCsTsGs**G**o**C**o**T**o**G**o**C**o**C**	62	1590-1609	3'-UTR
16843	**C**o**T**o**T**o**G**o**G**o**C**sTsGsCsAsGsGsCsCs**G**o**A**o**C**o**A**o**C**o**C**	63	1685-1704	3'-UTR
16844	**C**o**G**o**G**o**C**o**C**o**A**sAsTsGsCsCsAsCsCs**A**o**C**o**A**o**G**o**C**o**C**	64	1789-1808	3'-UTR
16845	**A**o**C**o**T**o**G**o**T**o**G**sCsTsCsCsTsGsCsTs**A**o**C**o**A**o**T**o**G**o**G**	65	1916-1935	3'-UTR
16846	**G**o**C**o**T**o**C**o**T**o**G**sGsCsCsAsGsCsAsGs**G**o**A**o**G**o**G**o**C**o**C**	66	1994-2013	3'-UTR
16847	**C**o**C**o**A**o**C**o**A**o**G**sCsCsAsGsCsCsTsGs**G**o**C**o**C**o**A**o**A**o**G**	67	2117-2136	3'-UTR
16848	**C**o**T**o**C**o**T**o**G**o**T**sCsTsTsCsGsTsGsAs**G**o**C**o**T**o**G**o**G**o**A**	68	2221-2240	3'-UTR
26264	**C**o**C**o**T**o**C**o**G**o**T**sGsCsTsGsCsGsGsCs**T**o**T**o**C**o**A**o**C**o**G**	69	mismatch	
26266	**C**o**C**o**T**o**G**o**G**o**T**sGsCsTsCsCsGsGsCs**T**o**T**o**C**o**A**o**C**o**G**	70	mismatch	
27693	**C**o**C**o**T**o**C**o**G**sTsGsGsTsGsCsGsCsCsTs**T**o**C**o**A**o**C**o**G**	54	0576-0595	coding
27694	**C**s**C**s**T**s**C**s**G**sTsGsGsTsGsCsGsCsCsTs**T**s**C**s**A**s**C**s**G**	54	0576-0595	coding

[1] Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy). All 2'-methoxyethoxy cytidines are 5-methyl-cytidines, underlined "C" residues are 5-methyl-cytidines; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester linkages.

[2] Coordinates from GenBank Accession No. U12597, locus name "HSU12597" SEQ ID NO.2.

HMVEC (human dermal microvascular) cells were purchased from Clonetics (San Diego CA) and cultivated in endothelial basal medium (EBM) supplemented with 10% fetal bovine serum (HyClone, Logan UT). Cells were grown in 100 mm petri dishes until 70-80% confluent and then treated with oligonucleotide in the presence of cationic lipid. Briefly, cells were washed with PBS and OPTI-MEM®. OPTI-MEM® containing 10 μg/mL LIPOFECTIN® was added to the cells, followed by addition of oligonucleotide. The cells were incubated for 3-4 hours at 37° C, washed once with EBM/1% FBS, and allowed to recover. For determination of mRNA levels by Northern blot, total RNA was prepared from cells by the guanidinium isothiocyanate procedure or by the Qiagen RNEASY™ method (Qiagen, Valencia, CA). Northern blot analysis was performed by standard methods (for example, Ausubel, et al. *Current Protocols in Molecular Biology,* Vol. 1, John Wiley and Sons, Inc., **1996**, pp.4.2.1-4.2.9). The probe was a PCR-labeled 1-kb fragment of TRAF-2 amplified by RT-PCR according to the method of Bednarczuk et al., *Biotechniques*, **1991**, 10,478. RNA was quantified and normalized to G3PDH mRNA levels using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager in accordance with manufacturer's instructions.

Results are shown in Table 4. Reduction of TRAF-2 mRNA levels with oligonucleotide 16834 (SEQ ID NO. 54) was determined to be dose-dependent in the range of 1 to 100 nM. The IC_{50} was approximately 10 nM. A TRAF-6 antisense oligonucleotide did not affect TRAF-2 mRNA expression.

The effect of oligonucleotide 16834 (SEQ ID NO. 54) on TRAF-2 protein levels was also examined. Cells were treated with oligonucleotide and allowed to recover for 48 to 72 hours before being harvested. Protein levels were determined by western blot analysis. Dose-dependent reduction of TRAF-2 protein expression was detectable 48

hours after treatment and maximal reduction of TRAF-2 protein levels was achieved 72 hours after treatment with 100 nM oligonucleotide.

TABLE 4

Activities of TRAF-2 Gapmer Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
LIPOFECTIN only	---	---	100%	0%
16827	47	5'-UTR	43%	57%
16828	48	5'-UTR	23%	77%
16829	49	AUG	48%	52%
16830	50	AUG	18%	82%
16831	51	coding	49%	51%
16832	52	coding	42%	58%
16833	53	coding	60%	40%
16834	54	coding	3%	97%
16835	55	coding	43%	57%
16836	56	coding	91%	9%
16837	57	coding	60%	40%
16838	58	coding	66%	34%
16839	59	coding	47%	53%
16840	60	coding	45%	55%
16841	61	coding	8%	92%
16842	62	3'-UTR	36%	64%
16843	63	3'-UTR	46%	54%
16844	64	3'-UTR	82%	18%
16845	65	3'-UTR	59%	41%
16846	66	3'-UTR	13%	87%
16847	67	3'-UTR	74%	26%
16848	68	3'-UTR	57%	43%

ISIS 27693 (SEQ ID NO: 54) was also shown to decrease TRAF-2 mRNA levels in primary human fibroblast-like synoviocytes (obtained from surgical/biopsy specimens). LIPOFECTIN® was included at 3 µg/ml. A dose-response effect was obtained with an IC_{50} of approximately 25 nM and nearly 90% reduction of TRAF-2 mRNA at an oligonucleotide concentration of 100 nM.

Example 17:

Antisense inhibition of TRAF-3 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target human TRAF-3 RNA using published sequences (GenBank accession number HSU21092, SEQ ID NO: 3. Oligodeoxynucleotides are shown in Table 5. Target sites are indicated as nucleotide numbers on the TRAF-3 mRNA target (SEQ ID NO: 3).

TABLE 5

Nucleotide Sequences of Human TRAF-3 Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
26778	AGAGCCGACGACCGCCGC	71	0078-0095	5'-UTR
26779	GGAAGAGCCGACGACCGC	72	0081-0098	5'-UTR
26780	CGCGCCAGGAGAGTCCAT	73	0236-0253	coding
26781	TTAGCGGCGGGTTAGTCT	74	0258-0275	coding
26782	AGCTTTAGCGGCGGGTTA	75	0262-0279	coding
26783	CTCGGTCTGCTTCGGGCT	76	0401-0418	coding
26784	TGCCCACACTCGGTCTGC	77	0409-0426	coding
26785	CGGTGCCCACACTCGGTC	78	0412-0429	coding

26786	GAAGCGGTGCCCACACTC	79	0416-0433	coding
26787	TTACACGCCTTCTCCACG	80	0712-0729	coding
26788	GTATTTACACGCCTTCTC	81	0716-0733	coding
26789	CCGGTATTTACACGCCTT	82	0719-0736	coding
26790	GAGGGCAGGACACCACCA	83	0816-0833	coding
26791	TGTGAGGGCAGGACACCA	84	0819-0836	coding
26792	CACTTGTGAGGGCAGGAC	85	0823-0840	coding
26793	GCTGGTTTGTCCCCTGAA	86	0939-0956	coding
26794	ATCTGCTGGTTTGTCCCC	87	0943-0960	coding
26795	CGCGGTTCTGGAGGGACT	88	1281-1298	coding
26796	CCCCGCACTCTTGTCCAC	89	1316-1333	coding
26797	TTGCCCCGCACTCTTGTC	90	1319-1336	coding
26798	CCACTTGCCCCGCACTCT	91	1323-1340	coding
26799	GAGCCACTTGCCCCGCAC	92	1326-1343	coding
26800	TTCCGAGCCACTTGCCCC	93	1330-1347	coding
26801	TCCGCCGCTTGTAGTCGC	94	1485-1502	coding
26802	TGCTTCCGCCGCTTGTAG	95	1489-1506	coding
26803	TCCTGCTTCCGCCGCTTG	96	1492-1509	coding
26804	GTCCCCGTTCAGGTAGAC	97	1589-1606	coding
26805	TCCCGTCCCCGTTCAGGT	98	1593-1610	coding
26806	CCATCCCGTCCCCGTTCA	99	1596-1613	coding
26807	TCCCCATCCCGTCCCCGT	100	1599-1616	coding
26808	CCCTTCCCCATCCCGTCC	101	1603-1620	coding
26809	TGCGTCCCCTTCCCCATC	102	1609-1626	coding
26810	AAGTGCGTCCCCTTCCCC	103	1612-1629	coding
26811	CGACAAGTGCGTCCCCTT	104	1616-1633	coding
26812	AAGGAAGCAGGGCATCAT	105	1662-1679	coding
26813	CTCTCCAGTGGGCTTCTT	106	1781-1798	coding
26814	TCATCTCTCCAGTGGGCT	107	1785-1802	coding
26815	GCTAAATCCACCTCCCCA	108	1933-1950	3'-UTR

26816	TCTGCCGCTTCCTCCGTC	109	2027-2044	3'-UTR
26817	CCGCCTTCTGCCGCTTCC	110	2033-2050	3'-UTR

[1] All cytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

[2] Coordinates from GenBank Accession No.U21092, locus name "HSU21092" SEQ ID NO.3.

Example 18:

Antisense inhibition of TRAF-3 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human TRAF-3 were synthesized. The oligonucleotide sequences are shown in Table 6. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. U21092), to which the oligonucleotide binds.

All compounds in Table 6 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

TABLE 6

Nucleotide Sequences of Human TRAF-3 Gapmer Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
26818	**AGAG**CCGACGACCG**CCGC**	71	0078-0095	5'-UTR
26819	**GGAA**GAGCCGACGA**CCGC**	72	0081-0098	5'-UTR
26820	**CGCG**CCAGGAGAGT**CCAT**	73	0236-0253	coding
26821	**TTAG**CGGCGGGTTA**GTCT**	74	0258-0275	coding
26822	**AGCT**TTAGCGGCGG**GTTA**	75	0262-0279	coding
26823	**CTCG**GTCTGCTTCG**GGCT**	76	0401-0418	coding
26824	**TGCC**CACACTCGGT**CTGC**	77	0409-0426	coding
26825	**CGGT**GCCCACACTC**GGTC**	78	0412-0429	coding
26826	**GAAG**CGGTGCCCAC**ACTC**	79	0416-0433	coding
26827	**TTAC**ACGCCTTCTC**CACG**	80	0712-0729	coding
26828	**GTAT**TTACACGCCT**TCTC**	81	0716-0733	coding
26829	**CCGG**TATTTACACG**CCTT**	82	0719-0736	coding
26830	**GAGG**GCAGGACACC**ACCA**	83	0816-0833	coding
26831	**TGTG**AGGGCAGGAC**ACCA**	84	0819-0836	coding
26832	**CACT**TGTGAGGGCA**GGAC**	85	0823-0840	coding
26833	**GCTG**GTTTGTCCCC**TGAA**	86	0939-0956	coding
26834	**ATCT**GCTGGTTTGT**CCCC**	87	0943-0960	coding
26835	**CGCG**GTTCTGGAGG**GACT**	88	1281-1298	coding
26836	**CCCC**GCACTCTTGT**CCAC**	89	1316-1333	coding
26837	**TTGC**CCCGCACTCT**TGTC**	90	1319-1336	coding
26838	**CCAC**TTGCCCCGCA**CTCT**	91	1323-1340	coding
26839	**GAGC**CACTTGCCCC**GCAC**	92	1326-1343	coding

26840	**TTCC**GAGCCACTTG**CCCC**	93	1330-1347	coding
26841	**TCCG**CCGCTTGTAG**TCGC**	94	1485-1502	coding
26842	**TGCT**TCCGCCGCTT**GTAG**	95	1489-1506	coding
26843	**TCCT**GCTTCCGCCG**CTTG**	96	1492-1509	coding
26844	**GTCC**CCGTTCAGGT**AGAC**	97	1589-1606	coding
26845	**TCCC**GTCCCCGTTC**AGGT**	98	1593-1610	coding
26846	**CCAT**CCCGTCCCCG**TTCA**	99	1596-1613	coding
26847	**TCCC**CATCCCGTCC**CCGT**	100	1599-1616	coding
26848	**CCCT**TCCCCATCCC**GTCC**	101	1603-1620	coding
26849	**TGCG**TCCCCTTCCC**CATC**	102	1609-1626	coding
26850	**AAGT**GCGTCCCCTT**CCCC**	103	1612-1629	coding
26851	**CGAC**AAGTGCGTCC**CCTT**	104	1616-1633	coding
26852	**AAGG**AAGCAGGGCA**TCAT**	105	1662-1679	coding
26853	**CTCT**CCAGTGGGCT**TCTT**	106	1781-1798	coding
26854	**TCAT**CTCTCCAGTG**GGCT**	107	1785-1802	coding
26855	**GCTA**AATCCACCTC**CCCA**	108	1933-1950	3'-UTR
26856	**TCTG**CCGCTTCCTC**CGTC**	109	2027-2044	3'-UTR
26857	**CCGC**CTTCTGCCGC**TTCC**	110	2033-2050	3'-UTR

[1] Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines and 2'-deoxycytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

[2] Coordinates from GenBank Accession No. U21092, locus name "HSU21092" SEQ ID NO.3.

Example 19

Antisense inhibition of TRAF-4 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human TRAF-4 RNA, using published sequences (GenBank accession number X80200, incorporated herein as SEQ ID NO: 4). The oligonucleotides are shown in Table 7. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. X80200), to which the oligonucleotide binds. All compounds in Table 7 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds are analyzed for effect on TRAF mRNA levels by quantitative real-time PCR as described in other examples herein.

TABLE 7

Nucleotide Sequences of Human TRAF-4 Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
26860	GCATGGCGGGCGAGCGGC	111	0072-0089	AUG
26861	CCGTCGCTTGGGCTTCTC	112	0113-0130	coding
26862	GGGCACTTGAAGACTCCT	113	0232-0249	coding
26863	CTCAGGGCACTTGAAGAC	114	0236-0253	coding
26864	TGGTCCTCAGGGCACTTG	115	0241-0258	coding
26865	AAGCTGGTCCTCAGGGCA	116	0245-0262	coding
26866	GCGGCAGCCCTCCTCACT	117	0341-0358	coding
26867	CTCCAGCGGCAGCCCTCC	118	0346-0363	coding
26868	TAGGGCAGGGAATGACAT	119	0411-0428	coding
26869	CGATTAGGGCAGGGAATG	120	0415-0432	coding

26870	GGGCAGCGATTAGGGCAG	121	0421-0438	coding
26871	GCCTCCCCACTGAAGTCA	122	0523-0540	coding
26872	ATGCGGGCACCACACTTA	123	0592-0609	coding
26873	GGGCAGGCAACAGGCAGC	124	0733-0750	coding
26874	CCACAGTGCCCACACCAC	125	0759-0776	coding
26875	CGAGCCACAGTGCCCACA	126	0763-0780	coding
26876	TCCTCCCGAGCCACAGTG	127	0769-0786	coding
26877	CAGGTCCTCCCGAGCCAC	128	0773-0790	coding
26878	GGCAGAGCACCAGGGCGG	129	0819-0836	coding
26879	CTTTGAATGGGCAGAGCA	130	0828-0845	coding
26880	GGAGTCTTTGAATGGGCA	131	0833-0850	coding
26881	ATGCCGTGCCATTGCCAG	132	0875-0892	coding
26882	CTCACCAGGGCACACATC	133	0925-0942	coding
26883	CAGCTCCTGCCGTTGCCG	134	0944-0961	coding
26884	ATGAGCACGCCATCACTG	135	1000-1017	coding
26885	TGTAGCCGCCGTCCATAG	136	1033-1050	coding
26886	GCCTCCTGTAGCCGCCGT	137	1039-1056	coding
26887	TAGAAGGCTGGGCTGAAG	138	1081-1098	coding
26888	GTGTGTAGAAGGCTGGGC	139	1086-1103	coding
26889	GTGTGCCCTCACCACTGC	140	1152-1169	coding
26890	GACACGGCGGGCAAAGGG	141	1226-1243	coding
26891	GAAGGTGACACGGCGGGC	142	1232-1249	coding
26892	GCCCAGGGTCGCTCTGAT	143	1260-1277	coding
26893	CTTCCAGTTTGGGTCGGG	144	1313-1330	coding
26894	GATAACCAAAGCCCAGAG	145	1377-1394	coding
26895	CATCGTCCTTTCCCCTCG	146	1513-1530	3'-UTR
26896	GGCCAGGGCTGAAGCACC	147	1660-1677	3'-UTR
26897	TTGTTTCCAGCCCTTCAT	148	1703-1720	3'-UTR
26898	CATGTCTGCCCTACCCAA	149	1746-1763	3'-UTR
26899	GCTCCCCTGCTGTGCCCT	150	1948-1965	3'-UTR

[1] All cytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

[2] Coordinates from GenBank Accession No. X80200, locus name "HSMLN62" SEQ ID NO. 4.

Example 20:

Antisense inhibition of TRAF-4 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human TRAF-4 were synthesized. The oligonucleotide sequences are shown in Table 8. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. X80200), to which the oligonucleotide binds.

All compounds in Table 8 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

Data are obtained by real-time quantitative PCR as described in other examples herein.

TABLE 8

Nucleotide Sequences of Human TRAF-4 Gapmer Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
26900	**GCAT**GGCGGGCGAG**CGGC**	111	0072-0089	AUG
26901	**CCGT**CGCTTGGGCT**TCTC**	112	0113-0130	coding
26902	**GGGC**ACTTGAAGAC**TCCT**	113	0232-0249	coding
26903	**CTCA**GGGCACTTGA**AGAC**	114	0236-0253	coding
26904	**TGGT**CCTCAGGGCA**CTTG**	115	0241-0258	coding
26905	**AAGC**TGGTCCTCAG**GGCA**	116	0245-0262	coding
26906	**GCGG**CAGCCCTCCT**CACT**	117	0341-0358	coding
26907	**CTCC**AGCGGCAGCC**CTCC**	118	0346-0363	coding
26908	**TAGG**GCAGGGAATG**ACAT**	119	0411-0428	coding
26909	**CGAT**TAGGGCAGGG**AATG**	120	0415-0432	coding
26910	**GGGC**AGCGATTAGG**GCAG**	121	0421-0438	coding
26911	**GCCT**CCCCACTGAA**GTCA**	122	0523-0540	coding
26912	**ATGC**GGGCACCACA**CTTA**	123	0592-0609	coding
26913	**GGGC**AGGCAACAGG**CAGC**	124	0733-0750	coding
26914	**CCAC**AGTGCCCACA**CCAC**	125	0759-0776	coding
26915	**CGAG**CCACAGTGCC**CACA**	126	0763-0780	coding
26916	**TCCT**CCCGAGCCAC**AGTG**	127	0769-0786	coding
26917	**CAGG**TCCTCCCGAG**CCAC**	128	0773-0790	coding
26918	**GGCA**GAGCACCAGG**GCGG**	129	0819-0836	coding
26919	**CTTT**GAATGGGCAG**AGCA**	130	0828-0845	coding
26920	**GGAG**TCTTTGAATG**GGCA**	131	0833-0850	coding
26921	**ATGC**CGTGCCATTG**CCAG**	132	0875-0892	coding
26922	**CTCA**CCAGGGCACA**CATC**	133	0925-0942	coding

26923	**CAGC**TCCTGCCGTT**GCCG**	134	0944-0961	coding
26924	**ATGA**GCACGCCATC**ACTG**	135	1000-1017	coding
26925	**TGTA**GCCGCCGTCC**ATAG**	136	1033-1050	coding
26926	**GCCT**CCTGTAGCCG**CCGT**	137	1039-1056	coding
26927	**TAGA**AGGCTGGGCT**GAAG**	138	1081-1098	coding
26928	**GTGT**GTAGAAGGCT**GGGC**	139	1086-1103	coding
26929	**GTGT**GCCCTCACCA**CTGC**	140	1152-1169	coding
26930	**GACA**CGGCGGGCAA**AGGG**	141	1226-1243	coding
26931	**GAAG**GTGACACGGC**GGGC**	142	1232-1249	coding
26932	**GCCC**AGGGTCGCTC**TGAT**	143	1260-1277	coding
26933	**CTTC**CAGTTTGGGT**CGGG**	144	1313-1330	coding
26934	**GATA**ACCAAAGCCC**AGAG**	145	1377-1394	coding
26935	**CATC**GTCCTTTCCC**CTCG**	146	1513-1530	3'-UTR
26936	**GGCC**AGGGCTGAAG**CACC**	147	1660-1677	3'-UTR
26937	**TTGT**TTCCAGCCCT**TCAT**	148	1703-1720	3'-UTR
26938	**CATG**TCTGCCCTAC**CCAA**	149	1746-1763	3'-UTR
26939	**GCTC**CCCTGCTGTG**CCCT**	150	1948-1965	3'-UTR

[1] Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines and 2'-deoxycytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

[2] Coordinates from GenBank Accession No. X80200, locus name "HSMLN62" SEQ ID NO. 4.

Example 21

Antisense inhibition of TRAF-5 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human TRAF-5 RNA, using published sequences (GenBank

accession number AB000509, incorporated herein as SEQ ID NO: 5). The oligonucleotides are shown in Table 9. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. AB000509), to which the oligonucleotide binds. All compounds in Table 9 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout.

TABLE 9

Nucleotide Sequences of Human TRAF-5 Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
26940	TGAATAAGCCATTGTGGG	151	0049-0066	AUG
26941	CTTTATGCTCTTCTGAAT	152	0062-0079	coding
26942	GGATGAAACCACAGGGCA	153	0083-0100	coding
26943	TCAAAGTCCAAGGAAATG	154	0120-0137	coding
26944	TGAAGCACCGAGTGGCAG	155	0195-0212	coding
26945	GGGCAGATTGGCACTGTG	156	0282-0299	coding
26946	CTCCTGAGATTTGATGAC	157	0313-0330	coding
26947	CTTTCCGTAGGACTGGCT	158	0491-0508	coding
26948	GATTCTGTAGATTGATGA	159	0584-0601	coding
26949	TTCATCTACCTCAGTTTT	160	0667-0684	coding
26950	TCCGTTACAGCACAGCCA	161	0735-0752	coding
26951	GCATGTGCTCCCGTAAGG	162	0788-0805	coding
26952	CTTTTCAAGTTTCTTTAT	163	0907-0924	coding

26953	CTTCCATCAAAGGTCTCA	164	1079-1096	coding
26954	TCTAAAACGGCTAATCTT	165	1146-1163	coding
26955	TCATCTTGTAATCTGTCA	166	1283-1300	coding
26956	GGACTGGCTGAAGATGGA	167	1333-1350	coding
26957	CCCTCCCTGACCCATCCC	168	1403-1420	coding
26958	GAATGAGCCACAAAGCGG	169	1620-1637	coding
26959	CAAGAACAGAGTGTCATC	170	1672-1689	coding
26960	GTCTAAATCCAGGTCAAT	171	1799-1816	3'-UTR
26961	AAACTTACCATCTTTCAA	172	1964-1981	3'-UTR
26962	CTCTGTGTCCTCCATAAC	173	2053-2070	3'-UTR
26963	CTTAACTGGAACAGCCTA	174	2167-2184	3'-UTR
26964	GCAGGAAGAATGAAAATG	175	2352-2369	3'-UTR
26965	TATTTGGTTGAATCTTAT	176	2501-2518	3'-UTR
26966	AAATTCTATCCATCCTCA	177	2611-2628	3'-UTR
26967	AAATTGTAAAGGTTTTCT	178	2683-2700	3'-UTR
26968	ACAATGAAACTCTGTCTC	179	2779-2796	3'-UTR
26969	GCAAAACTCCGTCTCTAC	180	2940-2957	3'-UTR
26970	CAATAGTTGTCAGAGGCT	181	3055-3072	3'-UTR
26971	AAGGACTCATCTCAGTTT	182	3209-3226	3'-UTR

26972	TAACAACGCAGAAGGGCT	183	3280-3297	3'-UTR
26973	AGTAGGGAAGTGGCATAA	184	3295-3312	3'-UTR
26974	CATCACCAGGTAAGCAGC	185	3377-3394	3'-UTR
26975	TCCTGTTGTGAACCTATT	186	3553-3570	3'-UTR
26976	GGACTTGTGGGCTAAAGA	187	3656-3673	3'-UTR
26977	GCTCAGGAAGACAGAGTG	188	3724-3741	3'-UTR
26978	TGAACTCCTAAGCAAACC	189	3873-3890	3'-UTR
26979	GATGATGAAGGAACTCTG	190	3889-3906	3'-UTR

[1] All cytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

[2] Coordinates from GenBank Accession No. AB000509, locus name "AB000509" SEQ ID NO. 5.

Example 22:

Antisense inhibition of TRAF-5 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human TRAF-5 were synthesized. The oligonucleotide sequences are shown in Table 10. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. AB000509), to which the oligonucleotide binds.

All compounds in Table 10 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

TABLE 10

Nucleotide Sequences of Human TRAF-5 Gapmer Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
26980	**TGAA**TAAGCCATTG**TGGG**	151	0049-0066	AUG
26981	**CTTT**ATGCTCTTCT**GAAT**	152	0062-0079	coding
26982	**GGAT**GAAACCACAG**GGCA**	153	0083-0100	coding
26983	**TCAA**AGTCCAAGGA**AATG**	154	0120-0137	coding
26984	**TGAA**GCACCGAGTG**GCAG**	155	0195-0212	coding
26985	**GGGC**AGATTGGCAC**TGTG**	156	0282-0299	coding
26986	**CTCC**TGAGATTTGA**TGAC**	157	0313-0330	coding
26987	**CTTT**CCGTAGGACT**GGCT**	158	0491-0508	coding
26988	**GATT**CTGTAGATTG**ATGA**	159	0584-0601	coding
26989	**TTCA**TCTACCTCAG**TTTT**	160	0667-0684	coding
26990	**TCCG**TTACAGCACA**GCCA**	161	0735-0752	coding
26991	**GCAT**GTGCTCCCGT**AAGG**	162	0788-0805	coding
26992	**CTTT**TCAAGTTTCT**TTAT**	163	0907-0924	coding

26993	**CTTC**CATCAAAGGT**CTCA**	164	1079-1096	coding
26994	**TCTA**AAACGGCTAA**TCTT**	165	1146-1163	coding
26995	**TCAT**CTTGTAATCT**GTCA**	166	1283-1300	coding
26996	**GGAC**TGGCTGAAGA**TGGA**	167	1333-1350	coding
26997	**CCCT**CCCTGACCCA**TCCC**	168	1403-1420	coding
26998	**GAAT**GAGCCACAAA**GCGG**	169	1620-1637	coding
26999	**CAAG**AACAGAGTGT**CATC**	170	1672-1689	coding
27000	**GTCT**AAATCCAGGT**CAAT**	171	1799-1816	3'-UTR
27001	**AAAC**TTACCATCTT**TCAA**	172	1964-1981	3'-UTR
27002	**CTCT**GTGTCCTCCA**TAAC**	173	2053-2070	3'-UTR
27003	**CTTA**ACTGGAACAG**CCTA**	174	2167-2184	3'-UTR
27004	**GCAG**GAAGAATGAA**AATG**	175	2352-2369	3'-UTR
27005	**TATT**TGGTTGAATC**TTAT**	176	2501-2518	3'-UTR
27006	**AAAT**TCTATCCATC**CTCA**	177	2611-2628	3'-UTR
27007	**AAAT**TGTAAAGGTT**TTCT**	178	2683-2700	3'-UTR
27008	**ACAA**TGAAACTCTG**TCTC**	179	2779-2796	3'-UTR
27009	**GCAA**AACTCCGTCT**CTAC**	180	2940-2957	3'-UTR
27010	**CAAT**AGTTGTCAGA**GGCT**	181	3055-3072	3'-UTR

27011	**AAGG**ACTCATCTCA**GTTT**	182	3209-3226	3'-UTR
27012	**TAAC**AACGCAGAAG**GGCT**	183	3280-3297	3'-UTR
27013	**AGTA**GGGAAGTGGC**ATAA**	184	3295-3312	3'-UTR
27014	**CATC**ACCAGGTAAG**CAGC**	185	3377-3394	3'-UTR
27015	**TCCT**GTTGTGAACC**TATT**	186	3553-3570	3'-UTR
27016	**GGAC**TTGTGGGCTA**AAGA**	187	3656-3673	3'-UTR
27017	**GCTC**AGGAAGACAG**AGTG**	188	3724-3741	3'-UTR
27018	**TGAA**CTCCTAAGCA**AACC**	189	3873-3890	3'-UTR
27019	**GATG**ATGAAGGAAC**TCTG**	190	3889-3906	3'-UTR

[1] Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines and 2'-deoxycytidines are 5-methyl-cytidines; all linkages are phosphorothioate linkages.

[2] Coordinates from GenBank Accession No. AB000509, locus name "AB000509" SEQ ID NO. 5.

Example 23:

Antisense inhibition of TRAF-6 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a series of oligonucleotides targeted to human TRAF-6 were synthesized. The oligonucleotide sequences are shown in Table 11. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. U78798), to which the oligonucleotide binds.

All compounds in Table 11 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of eight 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by six-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) in the "deoxy gap" and phosphodiester (P=O) in the wings. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

TABLE 11:

Nucleotide Sequences of TRAF-6 Gapmer Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE COORDINATES[2]	GENE TARGET REGION
15779	AoGoGoCoCoCoAsAsGsCsCsCsCsAsGsCoToGoCoGoG	191	0001-0020	5'-UTR
15880	CoGoCoCoAoCsCsTsTsCsGsCsTsGsGoCoCoGoCoC	192	0024-0043	5'-UTR
15881	GoAoGoAoCoGsAsGsGsCsTsGsCsTsToGoGoAoCoG	193	0071-0090	5'-UTR
15882	GoGoAoCoAoCsAsGsAsCsAsCsTsGsCoGoCoGoCoC	194	0091-0110	5'-UTR
15883	CoCoAoAoGoGsCsGsCsTsGsGsTsAsGoAoGoGoAoC	195	0111-0130	5'-UTR
15884	ToToGoCoToCsGsTsTsCsTsAsGsTsGoCoGoCoGoG	196	0185-0204	5'-UTR
15885	CoAoToAoGoTsAsAsCsTsTsGsAsTsToAoToCoAoC	197	0205-0224	AUG
15886	AoGoCoAoGoAsCsTsCsAsTsAsGsTsAoAoCoToToG	198	0213-0232	AUG
15887	AoCoAoGoToTsTsAsGsCsAsGsAsCsToCoAoToAoG	199	0220-0239	AUG
15888	AoCoAoGoCoGsCsTsAsCsAsGsGsAsGoCoToGoGoC	200	0291-0310	coding
15889	AoToToGoAoTsTsTsTsAsTsGsAsTsGoCoAoGoGoC	201	0495-0514	coding
15890	GoToGoAoCoCsTsGsCsAsTsCsCsCsToToAoToToG	202	0511-0530	coding
15891	GoToCoToCoAsGsTsTsCsCsAsTsCsToToGoToGoC	203	0641-0660	coding
15892	AoGoAoGoCoAsAsAsCsTsCsAsCsAsAoToGoToGoC	204	0678-0697	coding

15893	ToToToToGoGsAsAsGsGsGsAsCsGsCoToGoGoCoA	205	0714-0733	coding
15894	AoAoAoToGoCsCsAsTsTsGsAsTsGsCoAoGoCoAoC	206	0796-0815	coding
15895	AoToToCoAoCsAsGsAsTsGsAsCsAsToToToGoCoC	207	0851-0870	coding
15896	CoGoToGoCoCsAsAsGsTsGsAsTsTsCoCoToCoToG	208	0981-1000	coding
15897	GoGoToGoToTsCsTsCsTsTsGsTsAsGoGoToGoGoC	209	1000-1019	coding
15898	GoGoCoCoAoAsCsAsTsTsCsTsCsAsToGoToGoToG	210	1024-1043	coding
15899	CoGoCoToCoAsAsAsCsTsAsTsGsAsAoCoAoGoCoC	211	1046-1065	coding
15900	AoGoGoCoGoAsCsCsCsTsCsTsAsAsCoToGoGoToG	212	1119-1138	coding
15901	CoCoAoToToTsTsAsGsCsAsGsTsCsAoGoCoToCoC	213	1163-1182	coding
15902	CoGoAoAoToGsGsTsTsCsGsTsTsTsGoAoGoCoToC	214	1206-1225	coding
15903	CoCoAoToToGsCsAsCsTsGsCsTsGsToGoCoToToC	215	1254-1273	coding
15904	GoCoAoGoToCsGsGsTsAsAsCsTsGsAoAoGoGoToG	216	1401-1420	coding
15905	GoCoCoToToAsCsAsGsGsTsGsCsTsToCoAoGoAoC	217	1532-1551	coding
15906	AoGoCoAoAoGsCsAsGsCsTsCsTsGsGoToToToGoG	218	1576-1595	coding
15907	GoGoCoToAoCsCsCsAsTsGsTsCsAsAoAoGoCoGoG	219	1724-1743	coding
15908	ToToGoToToTsTsTsGsAsGsCsAsAsGoToGoAoGoG	220	1796-1815	3'-UTR
15909	GoGoCoAoCoTsGsTsTsTsTsCsTsCsCoAoGoGoToA	221	1817-1836	3'-UTR
15910	AoCoAoToAoTsTsTsCsCsCsGsTsGsGoCoToToGoT	222	1871-1890	3'-UTR

15911	GoGoAoAoCoGsTsGsTsGsGsAsTsTsCoCoCoAoGoG	223	1967-1986	3'-UTR
15912	ToGoCoToGoCsAsAsCsAsTsGsCsCsAoCoAoGoGoC	224	2017-2036	3'-UTR
15913	AoToAoCoAoCsCsAsGsAsGsCsAsAsAoAoGoCoCoC	225	2078-2097	3'-UTR
15914	AoAoAoAoGoAsCsTsGsAsAsCsTsTsToToAoAoGoG	226	scrambled control	
23247	AoCoToToAoAsTsTsAsCsCsAsTsGsAoCoToAoGoT	227	15910 mismatch	
23248	CoCoAoCoGoAsGsGsAsGsCsAsCsCsAoToCoAoAoG	228	16834 mismatch	
27691	AsCsAsTsAsTsTsTsCsCsCsGsTsGsGsCsTsTsGsT	222	1871-1890	3'-UTR
27692	AoCoAoToAsTsTsTsCsCsCsGsTsGsGsCoToToGoT	222	1871-1890	3'-UTR

[1] Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy). All 2'-methoxyethoxy cytidines are 5-methyl-cytidines, underlined "C" are 5-methyl-cytidine; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester linkages.

[2] Coordinates from GenBank Accession No. U78798, locus name "HSU78798" SEQ ID NO. 6.

HMVEC cells were grown in 100 mm petri dishes until 70-80% confluent and then treated with oligonucleotide in the presence of cationic lipid. Briefly, cells were washed with PBS and OPTI-MEM®. OPTI-MEM® containing 10 µg/mL LIPOFECTIN® (Life Technologies, Rockville MD) was added to the cells, followed by addition of oligonucleotide. The cells were incubated for 3-4 hours at 37° C, washed once with EBM/1% FBS, and allowed to recover. For determination of mRNA levels by Northern blot, total RNA was prepared from cells by the guanidinium isothiocyanate procedure or by the Qiagen RNEASY™ method (Qiagen, Valencia, CA). Northern blot analysis was performed by standard methods (for example, Ausubel, et al. *Current Protocols in Molecular Biology,* Vol. 1, John Wiley and Sons, Inc., **1996**, pp.4.2.1-4.2.9). The probe was a PCR-labeled 1-kb fragment of TRAF-6 amplified by RT-PCR according to the method of Bednarczuk *et al.*, **1991**, Biotechniques *10*,478. RNA was quantified and normalized to G3PDH mRNA levels using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager in accordance with manufacturer's instructions.

Results are shown in Table 12. Reduction of TRAF-6 mRNA levels with oligonucleotide 15910 (SEQ ID NO. 224) was determined to be dose-dependent in the range of 1 to 100 nM. The IC_{50} was approximately 2.5 nM. A TRAF-2 antisense oligonucleotide did not affect TRAF-6 mRNA expression.

TABLE 12

Activities of TRAF-6 Gapmer Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
LIPOFECTIN® only	---	---	100%	0%
15779	191	5'-UTR	62%	38%

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
15880	192	5'-UTR	73%	27%
15881	193	5'-UTR	28%	72%
15882	194	5'-UTR	96%	4%
15883	195	5'-UTR	57%	43%
15884	196	5'-UTR	73%	27%
15885	197	AUG	61%	39%
15886	198	AUG	37%	63%
15887	199	AUG	23%	77%
15888	200	coding	31%	69%
15889	201	coding	42%	58%
15890	202	coding	49%	51%
15891	203	coding	50%	50%
15892	204	coding	32%	68%
15893	205	coding	18%	82%
15894	206	coding	43%	57%
15895	207	coding	41%	59%
15896	208	coding	20%	80%
15897	209	coding	60%	40%
15898	210	coding	23%	77%
15899	211	coding	66%	34%
15900	212	coding	54%	46%
15901	213	coding	60%	40%
15902	214	coding	76%	24%
15903	215	coding	58%	42%
15904	216	coding	77%	23%
15905	217	coding	108%	---
15906	218	coding	90%	10%

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
15907	219	coding	62%	38%
15908	220	3'-UTR	82%	18%
15909	221	3'-UTR	28%	72%
15910	222	3'-UTR	13%	87%
15911	223	3'-UTR	103%	---
15912	224	3'-UTR	20%	80%
15913	225	3'-UTR	97%	3%
15914	226	scrambled control	70%	30%

Example 24

Effect of inhibiting TRAF Gene Expression on the Induction of E-selectin

The effect of TRAF antisense oligonucleotides on the induction of E-selectin by TNFα or IL-1β was examined. HMVEC cells were treated with either ISIS 16834 or ISIS 15910 under dose-response conditions followed by stimulation of E-selectin expression by TNFα or IL-1β for 5 hours. The cell surface expression of E-selectin was determined by flow cytometry analysis. Dose-dependent inhibition of E-selectin cell surface induction by TNFα was observed in cells treated with the TRAF-2 antisense oligonucleotide ISIS 16834, as expected. Surprisingly, the TRAF-6 antisense compound, ISIS 15910, was able to inhibit TNFα mediated E-selectin surface expression as well, especially at higher dose. At low doses (20-50nM), ISIS 16834 was a more effective inhibitor of TNFα-mediated E-selectin induction than ISIS 15910. Maximal inhibition of E-selectin induction for both antisense compounds was approximately 70% at 100 nM. Control oligonucleotides

exhibited little to no effect on E-selectin induction. When IL-1β was used as the stimulator, however, ISIS 15910 appeared to be a more specific and potent inhibitor of E-selectin induction than ISIS 16834, especially at relatively low doses.

Example 25

Effect of TRAF Antisense Oligonucleotide on IκBα Phosphorylation and Degradation

Multiple transcription factors are activated by cytokines to facilitate the induction of E-selectin. The most important and best studied transcription factors involved in the regulation of E-selectin activation include NF-κB, c-Jun and ATF-2. To clarify the roles of TRAF proteins in the activation of NF-κB by cytokines, IκBα phosphorylation and degradation assays were performed with antisense oligonucleotide treated cells. Cells were treated with either ISIS 16834 or ISIS 15910 and allowed to recover for 48-72 hours. Tumor necrosis factor-α (TNF-α) or interleukin-1-β (IL-1β) was added for 5 to 30 minutes before cells were harvested. Western blot analysis with antibody specific for phospho-IκBα was performed to study the phosphorylation of IκBα. The blots were then stripped and reblotted with antibody against IκBα to study the degradation of IκBα. IκBα was heavily phosphorylated 5 minutes after addition of either cytokine. By 30 minutes, IκBα was reduced, probably as a result of IκBα degradation. In TNFα-stimulated cells, the majority of the IκBα had been degraded after 5 minutes of stimulation. By 30 minutes, IκBα was almost completely gone. In contrast, the degradation of IκBα in IL-1β stimulated cells was slower with the majority of IκBα degraded by 30 minutes. Neither ISIS 16834 nor ISIS 15910 affected IκBα phosphorylation and degradation induced by TNFα. ISIS 15910 has little effect

on IL-1β mediated IκBα phosphorylation and degradation either. Hyperphosphorylation of IκBα was observed in ISIS 16834 treated, IL-1β induced cells. In summary, the antisense oligonucleotides do not inhibit the phosphorylation and degradation of IκBα.

Example 26

Effect of TRAF Antisense Oligonucleotides on JNK Activities

MAP kinases play central roles in the activation of specific transcription factors crucial to the induction of cell adhesion molecules. To examine the effect of TRAF antisense oligonucleotides on JNK activities, *in vitro* kinase assays were performed on extracts derived from cells treated with TRAF antisense oligonucleotides. Cells were treated with TRAF-2 or TRAF-6 antisense compounds, (ISIS 16834 or ISIS 15910, respectively) allowed to recover for 48-72 hours, at which time TNF was added for 15 minutes prior to the cell lysis and the initiation of the kinase assays. Specific c-Jun conjugated agarose beads were used to precipitate JNK. ATP was added to the immunoprecipitated kinase complexes and the reaction mixes were analyzed on SDS-PAGE. Western blotting with antibodies specific for phosphorylated c-Jun was carried out to determine relative kinase activity. JNK was activated by TNFα after a 15 minute incubation, as indicated by the heavy phosphorylation of c-Jun. ISIS 16834 reduced JNK activity in TNFα-treated cells but not in IL-1β treated cells. Some hyperphosphorylation of c-Jun induced by IL-1β in ISIS 16834 treated cells was observed. ISIS 15910 reduced the c-Jun phosphorylation mediated by both IL-1β and TNFα. Some inhibitory effect of ISIS 15910 on JNK activity was also observed in TNFα-induced cells. This result is consistent with the inhibitory effects of

TRAF antisense oligonucleotides on the surface expression of E-selectin.